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# Single-Tube, Nested, Reverse Transcriptase PCR for Detection of Viable *Mycobacterium tuberculosis*

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**Several problems remain before molecular biology-based techniques, such as PCR, are widely accepted for the detection of infectious agents. Among the most formidable of these problems are the inability of the tests to distinguish between viable and nonviable organisms. We approached this problem by using the fact that bacterial mRNA has an extremely short half-life, averaging only a few minutes. We reasoned that by targeting bacterial mRNA by a reverse transcriptase PCR (RT-PCR), a positive signal would indicate the presence of a recently viable organism. To test our hypothesis, we chose to target the mRNA coding for the ubiquitous 85B antigen of mycobacteria. After partially sequencing the gene coding for 85B, we developed primers that were specific for *Mycobacterium tuberculosis*. In a single-tube, nested, RT-PCR (STN RT-PCR), these primers detected fewer than 40 CFU in spiked sputum samples and as few as 12 CFU in clinical sputum specimens. The sensitivity of STN RT-PCR with smear-negative samples was as good as that of culture. The specificity was 100%. More importantly, when *M. tuberculosis* was cultured with and without 1 μg of isoniazid per ml, this assay could distinguish between those cultures which contained the antibiotic and those which did not. Subcultures on Lowenstein-Jensen agar confirmed the viability assessments of the STN RT-PCR. Control experiments demonstrated that isoniazid did not inhibit the RT-PCR. In addition, when an IS*6110*-targeted, DNA PCR was used to examine the same samples, all samples through 13 days (the last sample) continued to be positive, irrespective of whether isoniazid was present, thereby demonstrating the superiority of an mRNA target in the detection of mycobacterial viability.**

The resurgence of tuberculosis has created a global public health emergency, propelled in large measure by the AIDS epidemic (10). Standard methods of diagnosis have proven to be inadequate for the task of swift detection and therapy of these infections, particularly in AIDS patients (2, 10). Moreover, this situation is worsened by an increasing number of patients with drug-resistant cases of infection (2). Because of these difficulties, PCR has become a popular method of detecting *Mycobacterium tuberculosis*. However, several issues must be resolved before PCR can be widely accepted for clinical use with specimens from patients with tuberculosis. These have been summarized by Bates (3): "The problems with any PCR method for the diagnosis of tuberculosis will include the risk of obtaining false-positive results due to contamination of clinical specimens with *M. tuberculosis* DNA product from the PCR laboratory, the inability of the PCR method to detect a difference between viable and nonviable organisms, and the inability of the PCR method to determine drug susceptibility." Considerable work has already gone into resolving the problem of contamination (5, 13, 14). Therefore, we have focused on the issues of assessing microbial viability and drug susceptibility using PCR.

To address these questions, we took advantage of the fact that the average half-life of bacterial mRNA is 3 min (1). Because mRNA is more rapidly destroyed in cells than rRNA or genomic DNA, we reasoned that an assay targeting bacterial mRNA would provide a better guide to mycobacterial viability than amplification tests directed at DNA or rRNA targets. Similarly, if it could distinguish viable from nonviable organisms, the same test should be able to detect the effects of antibiotics on these organisms and, thus, rapidly determine antibiotic susceptibility or resistance.

We chose as a target for amplification the mRNA coding for 85B, one of three homologous proteins that are part of the 85 antigen complex of mycobacteria. This protein is known to be secreted in large quantities from growing mycobacteria (19) and thus should represent a relatively abundant target. The 85 antigen complex is present in all mycobacteria tested (7, 21), and there is considerable evidence that the complex contains both species-specific and shared epitopes (18). Thus, this antigen would provide a target which is universally present but which still permits species differentiation by primer manipulation.

## MATERIALS AND METHODS

**Mycobacterial cultures.** *M. tuberculosis* H37Rv (ATCC 27294) was cultured in 7H9 broth for 7 to 14 days. Aliquots were shaken with five 3-mm-diameter Kimax glass beads (VWR Scientific, Cerritos, Calif.) for 5 to 10 min to facilitate dispersion, and the aliquots were diluted so that their turbidities were equivalent to that of a McFarland no. 1 standard in 7H9 broth. Further dilutions were made as specified in individual experiments.

Various strains of mycobacteria including *M. bovis*, *M. avium*, *M. kansasii*, *M. scrofulaceum*, *M. microti*, and *M. marinum*, as well as photochromes and scotochromes, were obtained from the Mycobacteriology Laboratory at Harbor-UCLA Medical Center.

After processing as described below, all sputa were cultured on Lowenstein-Jensen slants, a Mycobactosel slant, and a Wallenstein slant.

**Sputum processing.** For those experiments which involved human sputum, residual, purulent sputum samples were obtained from the Harbor-UCLA Medical Center Microbiology Laboratory and were treated with the mucolytic agent Sputolysin (dithiothreitol) for 15 min. The samples were then incubated with equal volumes of 4% NaOH containing phenolphthalein for exactly 20 min and were back-titrated to neutral with 5% oxalic acid. The treated samples were centrifuged at 3,000 × *g* for 15 min, and the pellets were examined by fluorescence microscopy (8). The residual pellet was immediately sent for nucleic acid extraction or was pooled and refrigerated at 4°C for no more than 3 days prior to use in experiments directed at determining the limits of detection of the single-tube, nested, reverse transcriptase PCR (STN RT-PCR) (see below).

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TABLE 1. PCR primers used in the study

| Oligonucleotides | Sequence (5′ 3′) | Melting temp (°C)[a] |
|---|---|---|
| *M. tuberculosis* IS*6110* primers for STN PCR | | |
| MRL29 | GGACAACGCCGAATTGCGAAGGGC | 78 |
| MRL30 | TAGGCGTCGGTGACAAAGGCCACG | 78 |
| MRL31 | CCATCGACCTACTACGACC | 60 |
| MRL32 | CCGAGTTTGGTCATCAGCC | 60 |
| *M. tuberculosis* 85B primers for STN RT-PCR | | |
| MRL41 | GAGTACCTGCAGGTGCCGTCGCCGTC | 88 |
| MRL42 | CCGGGTGTTGTTTGCGACCAGCTTG | 80 |
| MRL43 | GACTTACAAGTGGGAAACC | 56 |
| MRL44 | CCGATCAGGCTAGGCCCC | 62 |

[a] [4(G + C) + 2(A + T)].

**RNA and DNA extraction.** Lysis of the mycobacteria required a two-step process. Bacterial or sputum pellets were first incubated with 250 μl of lysozyme buffer (lysozyme at 10 mg/ml, 50 mM glucose, 25 mM Tris-HCl [pH 8.0], and 10 mM EDTA [pH 8.0]) for 30 min on ice. This was followed by incubation with 250 μl of proteinase K buffer containing proteinase K (CalBiochem, La Jolla, Calif.) at 2 mg/ml, 2% sodium dodecyl sulfate, and 3% diethyl pyrocarbonate for 1 h at 50°C. All reagents were prepared as RNase-free solutions with diethyl pyrocarbonate-treated water.

RNA was purified from the aqueous phase by acid phenol-chloroform (5:1) extraction (pH 4.7) and ethanol precipitation in the presence of tRNA (1 mg/ml) as a carrier (4). Twenty units of DNase I and $MgCl_2$ at a final concentration of 0.01 M were added to the extracted RNA (15). The material was incubated at 37°C for 30 min to digest the DNA. The RNA was purified again by acid phenol-chloroform extraction and ethanol precipitation.

DNA was purified from the organic phase generated during RNA extraction. Briefly, 1 volume of STE buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) containing 1% sodium dodecyl sulfate and adjusted to pH 12.0 with 5 N NaOH was added to the organic phase (4). After vortexing for 30 s and centrifuging at 12,000 × *g* for 20 min, the DNA was isolated from the aqueous phase by ethanol precipitation in the presence of 10 μg of salmon sperm DNA. When not used immediately, the purified DNA and RNA were aliquoted and stored at −70°C.

**Partial sequencing the 85B antigen-coding region for *M. tuberculosis*.** The 85B antigen-coding sequence has previously been determined for *M. bovis* and *M. kansasii* (16, 17). We used conserved segments of the 85B gene to develop primers which would amplify DNA from *M. tuberculosis* H37Rv. These same oligonucleotides were used as sequencing primers with the dsDNA Cycle Sequencing System as described by the manufacturer (Gibco BRL, Grand Island, N.Y.). The primers used for sequencing (MRL41 and MRL42) as well as their reported positions in the *M. bovis* sequence are indicated in Table 1.

**STN PCR.** Amplification was performed in 0.5-ml thin-walled PCR tubes with a total reaction volume of 50 μl by using a model 480 thermal cycler (Perkin-Elmer, Foster City, Calif.). The oligonucleotide primers used for amplification of the *M. tuberculosis* DNA target sequences in the IS*6110* insertion element are indicated in Table 1. The outer and inner primers for *M. tuberculosis* were designated primers MRL29 and MRL30 and primers MRL31 and MRL32, respectively. They have been modified from those used by Wilson et al. (20) to permit two-cycle rather than three-cycle amplification in the first phase of the STN PCR (20).

The amplification reactions contained the individual outer and inner primers at final concentrations of 0.01 and 1 μM, respectively, 2.5 U of *Taq* polymerase (Perkin-Elmer) in amplification buffer, 200 μM (each) dATP, dCTP, and dGTP and 600 μM dUTP (Perkin-Elmer), and 0.5 U of thermolabile uracil *N*-glycosylase (HK-UNG; Epicenter Technologies, Madison, Wis.) to control cross contamination (5). After a 10-min incubation at 50°C to allow the HK-UNG to work, the temperature was raised to 94°C for 2 min to inactivate the enzyme and break up any contaminating DNA.

The thermal cycler was programmed to carry out the DNA PCR in two stages. The first stage, for the outer primers, involved 30 cycles of denaturation at 94°C for 45 s, with primer annealing and extension carried out in one step at 70°C for 1.5 min. The second stage, for the inner primers, included 30 cycles of denaturation at 94°C for 45 s, primer annealing at 60°C for 45 s, and extension at 72°C for 45 s, after which the reaction mixture was held at 72°C for 30 min. Ten microliters of the amplification product was electrophoresed on 3% NuSieve GTG–1% agarose gels, stained with 1 μg of ethidium bromide per ml, and visualized by UV transillumination. The presence of a 197-bp band indicated successful amplification of the IS*6110* target.

**STN RT-PCR.** Recombinant *Thermus thermophilus* (r*Tth*) DNA polymerase was used (GeneAmp EZ rTth RNA PCR Kit; Perkin-Elmer). The oligonucleotide primers used for amplification of the *M. tuberculosis* 85B mRNA target sequences are indicated in Table 1. The outer and inner primers for *M. tuberculosis* are designated primers MRL41 and MRL42 and primers MRL43 and MRL44, respectively. Their positions in the 85B sequence are indicated in Fig. 1.

The amplification reactions contained the individual outer and inner primers at final concentrations of 0.01 and 0.8 μM, respectively, 5 U of r*Tth* DNA polymerase, 200 μM (each) dATP, dCTP, and dGTP, 600 μM dUTP, 0.5 U of HK-UNG, 10 μl of 5× EZ buffer, 5.0 μl of 25 mM manganese diacetate, and a quantity of water sufficient to bring the volume to 50 μl. After a 10-min incubation at 50°C to allow the HK-UNG to work, the temperature was raised to 60°C for 30 min to initiate synthesis of the first cDNA strand and then to 94°C for 2 min to inactivate the HK-UNG. The first and second stages of the amplification were then conducted as described above. The presence of a 216-bp signal on agarose gel electrophoresis indicated successful amplification of the 85B target.

## RESULTS

**Primer selection and specificity.** The partial coding sequence of 85B determined from *M. tuberculosis* DNA demonstrated a few differences from that published for *M. bovis* (Fig. 1). One such difference consisted of an insertion of six bases (GCCTAG) between positions 689 and 690 of the published sequence (19). We exploited this difference to produce primers which distinguished between *M. tuberculosis* and other mycobacteria, even closely related species such as *M. bovis*. The set of nested primers that were selected successfully produced a 216-bp amplification product from *M. tuberculosis* DNA. These primers were specific and proved to be capable of distinguishing *M. tuberculosis* from 18 other species and strains of mycobacteria including *M. bovis*, *M. avium*, *M. kansasii*, *M. scrofulaceum*, *M. microti*, and *M. marinum*, as well as photochromes and scotochromes (data not shown). In addition, the sequences of the primers were compared to sequences in the GenBank and EMBL DNA libraries. No two primers corresponded to or complemented DNA registered in these libraries in a manner that would permit amplification.

**Limits of detection of the STN RT-PCR.** Seven-day, pure cultures of *M. tuberculosis* H37Rv underwent serial 10-fold dilutions with 7H9 medium. Aliquots of the dilutions were cultured on Lowenstein-Jensen slants to assess the number of bacilli present in the dilutions and their viabilities. Using the four primers described above, the STN RT-PCR detected 85B mRNA in dilutions of pure *M. tuberculosis* cultures that produced as few as 38 CFU from 0.1-ml culture aliquots on agar slants. With clinical sputum samples, the technique detected as few as one to nine bacilli per high-power field on fluorochrome staining and 12 CFU on culture (Fig. 2).

To further define the limits of detection of this assay, residual, purulent sputum samples from individuals not suspected of having tuberculosis were obtained by the hospital microbiology laboratory, pooled, decontaminated, treated with a mucolytic agent, and concentrated as described above. Fluoro-

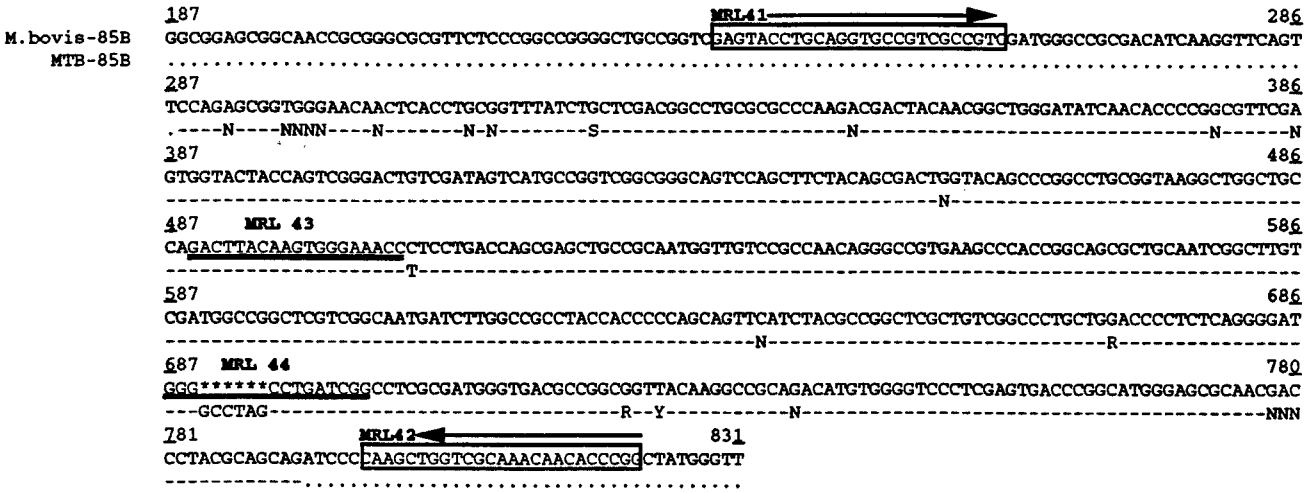


FIG. 1. Comparison of the DNA sequences of the 85B antigen in *M. bovis* BCG and *M. tuberculosis* H37Rv. The sequence for *M. tuberculosis* was compared and aligned with the published sequence for *M. bovis* BCG (12). Dashes, identical sequences; dots, sequences that have not been determined; N, unresolved residues; S, G or C; Y, C or T; R, A or G. Primers MRL41 and MRL42 are shown in enclosed boxes, while the underlined sequences represent primers MRL43 and MRL44. MRL44 contains the 6-base insertion not present in the *M. bovis* 85B sequence.

chrome staining and subsequent culture on Lowenstein-Jensen slants confirmed the lack of mycobacteria in the pooled samples.

Aliquots of the treated, pooled sputa were spiked with dilutions of a culture containing a known quantity of *M. tuberculosis* H37Rv. Samples were mixed by vortexing for 30 s. The RNA was extracted and subjected to STN RT-PCR with primers to detect the 85B mRNA targets. Simultaneous fluorochrome smears and cultures of the dilutions of *M. tuberculosis* on Lowenstein-Jensen agar served as additional controls for the number and viability of the mycobacteria detected by the PCR.

The results of one such experiment displayed in Table 2 demonstrate that the STN RT-PCR could detect between 1 and 10 bacilli. For smear-negative samples (1 to 1,000 bacilli/ml), this PCR assay was at least as sensitive as the single agar-based culture method (sensitivity of PCR, 83%; sensitivity of culture, 75%). The specificity of PCR was 100% (Table 2).

**Detection of mycobacterial viability by the STN RT-PCR.** To determine if the STN RT-PCR would detect living but not dead *M. tuberculosis* bacilli, isoniazid at 0.1 μg/ml was added to cultures of *M. tuberculosis* H37Rv in 7H9 medium but not to control cultures.

The STN RT-PCR was able to detect the difference between a culture which contained antibiotic and one which did not after 7 days of incubation (Fig. 3A). In an important control experiment, neither isoniazid nor rifampin inhibited the STN RT-PCR when they were added directly to the reaction mixture (data not shown).

When an IS*6110*-targeted, DNA PCR was used to examine the same samples, all samples through 13 days (the last sample) continued to be positive, irrespective of whether isoniazid was present (Fig. 3B). The viability (or lack thereof) of the organisms contained in these cultures was confirmed by simultaneous standard culture on Lowenstein-Jensen agar.

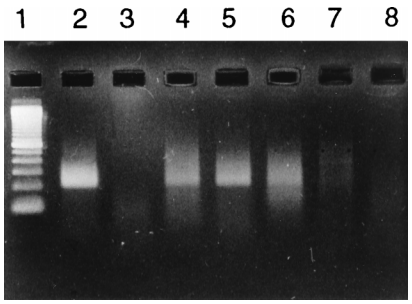


FIG. 2. STN RT-PCR can detect *M. tuberculosis* in spear-positive sputum. Lane 1, 100-bp DNA ladder; lane 2, positive control (216-bp amplification product of STN RT-PCR performed with RNA extracted from *M. tuberculosis* H37Rv); lane 3, no-target negative control; lanes 4 to 6, 216-bp amplification products from sputa containing 10 to 90, 10 to 90, and 1 to 9 *M. tuberculosis* isolates per high-power field, respectively, on fluorochrome staining; lanes 7 and 8, negative amplification of sputa containing no bacteria on fluorochrome staining. All spear-positive sputa were later culture positive; smear-negative sputa were culture negative.

## DISCUSSION

The STN RT-PCR procedure described in this report addresses all of the problems outlined by Bates (3) and noted above. One objective was to avoid contamination. The STN PCR was initially designed to avoid the inherent contamination potential of standard nested PCR without relinquishing the extremely powerful amplification potential of the nested format (20). To our knowledge, this is the first attempt to use this strategy with an RT-PCR. The addition of UNG provided

TABLE 2. Results of simultaneous smear, culture, and PCR for spiked sputum samples

| No. of bacilli/ml of sputum | Smear result[a] | Culture result[b] | PCR result |
|---|---|---|---|
| $1 \times 10^3$ | 0 | m | + |
| $5 \times 10^2$ | 0 | m | + |
| $1 \times 10^2$ | 0 | m | + |
| $5 \times 10^1$ | 0 | 3 | + |
| $1 \times 10^1$ | 0 | 0 | + |
| $1 \times 10^0$ | 0 | 0 | + |
| $1 \times 10^{-1}$ | 0 | 0 | − |
| 0 | 0 | 0 | − |

[a] Number of bacteria in 10 high-power fields on smear.
[b] Number of CFU; m, >50 CFU.

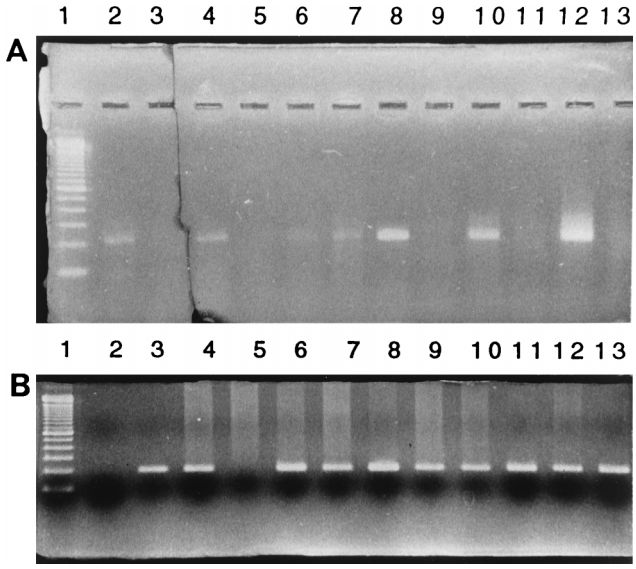


FIG. 3. (A) RT-PCR can distinguish cultures of *M. tuberculosis* containing isoniazid from those without isoniazid. Lane 1, a 100-bp ladder; lane 2, positive control (216-bp amplification product of STN RT-PCR performed with RNA extracted from *M. tuberculosis* H37Rv); lane 3, no-target negative control; lane 4, 216-bp amplification product in day 0 culture of H37R immediately after the addition of isoniazid; lane 5, day 0 medium-only control; lane 6, 216-bp amplification product from day 3 control culture without isoniazid; lane 7, 216-bp amplification product from day 3 control culture without isoniazid; lane 8, 216-bp amplification product from day 7 culture with isoniazid; lane 9, absence of 216 bp amplification product from day 7 culture containing isoniazid; lane 10, 216-bp amplification product from day 11 control culture without isoniazid; lane 11, absence of 216-bp amplification from day 11 culture containing isoniazid; lane 12, 216-bp amplification product from day 13 control culture without isoniazid; lane 13, absence of 216-bp amplification product from day 13 containing isoniazid. (B) IS*6110*-targeted, DNA PCR cannot distinguish cultures of *M. tuberculosis* containing isoniazid from those without isoniazid. Lane 1, a 100-bp ladder; lane 2, no-target negative control; lane 3, positive control (197-bp amplification product of STN PCR performed with DNA extracted from *M. tuberculosis*); lane 4, 197-bp amplification product in day 0 culture of H37Rv immediately after the addition of isoniazid; lane 5, day 0 medium-only control; lanes 6 and 7, 197-bp amplification product from day 3 cultures with isoniazid (lane 6) and without isoniazid (lane 7); lanes 8 and 9, 197-bp amplification product from day 7 cultures with isoniazid (lane 8) and without isoniazid (lane 9); lanes 10 and 11, 197-bp amplification product from day 11 cultures with isoniazid (lane 10) and without isoniazid (lane 11); lanes 12 and 13, 197-bp amplification product from day 13 cultures with isoniazid (lane 12) and without isoniazid (lane 13).

further protection against contamination (5). The use of UNG would normally be impossible with a nested format because its use in the second stage of the nested PCR would eliminate the amplified target generated in the first stage. Since our STN RT-PCR procedure is performed in a single tube and no transfers are necessary, the UNG can be inactivated prior to the initiation of amplification without sacrificing effective decontamination. Although UNG rapidly removes uracil from DNA, the target in this assay, mRNA, remains unaffected.

Our primary objective was to develop a PCR technique which could distinguish viable from nonviable tubercle bacilli. The STN RT-PCR accomplishes this (Fig. 3A) while still retaining sufficient sensitivity to detect the equivalent of smear-negative samples. It is equally clear that DNA-targeted PCR was incapable of determining mycobacterial viability over the time period tested. This is not a trivial distinction. Yuen and colleagues (22), who used an IS*6110* DNA target, reported that the sputa of 70% of 41 patients with proven tuberculosis had a positive PCR signal 4 weeks after the onset of therapy, whereas cultures were positive for only 32% of the cohort at the same point in time. Moreover, Eisenach (9) has described the detection of the same PCR target more than 500 days after the initiation of therapy and long after cultures were negative. Is this the detection of dead organisms, inadequate therapy, or merely cross contamination? These are extremely important questions that have considerable bearing on clinical decision making and the natural course of treated disease. The STN RT-PCR should be able to resolve these questions.

Because the STN RT-PCR can rapidly detect the effect of antibiotics on mycobacterial viability (Fig. 3A), it may be possible to design a PCR-based system that can be used to quickly perform antibiotic susceptibility testing. Our experiments document that STN RT-PCR can detect mycobacterial susceptibility to isoniazid in 4 to 7 days when an initial inoculum of 20,000 to 50,000 organisms/ml is used. Recently, a survey of state microbiology laboratories sponsored by the Centers for Disease Control and Prevention noted that even those which use rapid radiometric techniques for both culture and susceptibility testing still require, on average, 31 days from the time of specimen processing to the time that the mycobacterial drug susceptibility result is reported (11). Even if a PCR-based system took 10 days to determine antibiotic susceptibilities, it would be a significant improvement. This would be particularly true in the case of smear-negative, STN RT-PCR-positive samples, for which there would be an even greater delay by standard techniques.

What these experiments do not address is whether the STN RT-PCR system can deal with the more difficult problem of assessing antibiotic resistance. A method would have to be developed to detect samples that contained more resistant bacilli than the current cutoff for resistance, i.e., more than 1% of the total inoculum.

Although the STN RT-PCR appears to demonstrate detection limits that are clinically acceptable, its sensitivity and specificity with a large number of clinical sputum samples containing a variety of potential PCR inhibitors are unknown. Moreover, the RNA extraction procedures used in the present studies are cumbersome and would probably require modification to be clinically practical. Whether this can be accomplished without compromising detection limits remains to be determined.

Finally, although we used STN RT-PCR, other RNA-based amplification systems such as transcription-mediated amplification or nucleic acid sequence-based amplification should be capable of producing similar results with an mRNA target (6, 12). Indeed, it is the utility of detecting an mRNA target to establish bacterial viability that we wish to emphasize here, rather than the particular method used to do so.

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